Trioplex Real-time RT-PCR Assay

Centers for Disease Control and Prevention

For use under an Emergency Use Authorization only

Instructions for Use

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Introduction

PURPOSE

This document describes the use of real-time (TaqMan®) RT-PCR assays for detection and differentiation of RNA from dengue, chikungunya and Zika viruses in serum, cerebrospinal fluid (CSF) and for the detection of Zika virus RNA in urine and amniotic fluid. This protocol has been designed to facilitate simultaneous testing for the presence of dengue, chikungunya and Zika viruses using a single sample.

NOTE: In this assay (Trioplex Real-time RT-PCR) the tests for dengue, chikungunya and Zika viruses are run in the same rRT-PCR plate well (multiplex).

INTENDED USE

The Trioplex Real-time RT-PCR Assay (Trioplex rRT-PCR) is intended for the qualitative detection and differentiation of RNA from Zika virus, dengue virus, and chikungunya virus in human sera or cerebrospinal fluid (collected alongside a patient-matched serum specimen), and for the qualitative detection of Zika virus RNA in urine and amniotic fluid (each collected alongside a patient-matched serum specimen). The assay is intended for use with specimens collected from individuals meeting CDC Zika virus clinical criteria (e.g., clinical signs and symptoms associated with Zika virus infection) and/or CDC Zika virus epidemiological criteria (e.g., history of residence in or travel to a geographic region with active Zika transmission at the time of travel, or other epidemiologic criteria for which Zika virus testing may be indicated as part of a public health investigation). Testing is limited to qualified laboratories designated by the Centers for Disease Control and Prevention (CDC).

Assay results are for the identification of Zika, dengue, and chikungunya viral RNA. Viral RNA is generally detectable in serum during the acute phase of infection (approximately 7 days following onset of symptoms, if present). Positive results are indicative of current infection. Laboratories are required to report all results to the appropriate public health authorities. Within the United States and its territories results must be reported to CDC.

Negative Trioplex rRT-PCR results do not rule out dengue, chikungunya and/or Zika virus infections and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

The Trioplex rRT-PCR is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

PROTOCOL USE LIMITATIONS

The Trioplex Real-Time RT-PCR Assay described here has not been extensively tested with clinical specimens. Modifications of these assays (i.e., use of PCR instruments or chemistries other than those described) is not permitted. These assays should not be further distributed without the explicit consent of the CDC.

ASSAY PRINCIPLE

Trioplex Real-Time RT-PCR Assay includes primers and dual-labeled hydrolysis (Taqman®) probes to be used in the *in vitro* qualitative detection of Zika virus RNA isolated from clinical specimens including serum (from serum separator tubes), CSF, urine, and amniotic fluid. A reverse transcription step produces cDNA from RNA present in the sample. The probe binds to the target DNA between the two unlabeled PCR primers. For the dengue virus-specific probe, the signal from the fluorescent dye (FAM) on the 5' end is quenched by BHQ-1 on its 3' end. For the chikungunya virus-specific probe, the signal from the fluorescent dye (HEX) on the 5' end is quenched by BHQ-1 on its 3' end. For the Zika virus-specific probe, the signal from the fluorescent dye (Texas Red [TxRd]) on the 5'end is quenched by BHQ-2 on its 3' end. During PCR, Taq polymerase extends the unlabeled primers using the template strand as a guide, and when it reaches the probe it cleaves the probe separating the dye from the quencher allowing it to fluoresce. The real-time PCR instrument detects this fluorescence from the unquenched dye. With each cycle of PCR, more probes are cleaved resulting in an increase in fluorescence that is proportional to the amount of target nucleic acid present.

Specimens

ACCEPTABLE SPECIMENS

For Zika, chikungunya and dengue testing:

- Serum (collected in a serum separator tube)
  Tube should be centrifuged prior to shipping to avoid hemolysis
- Cerebrospinal fluid

For Zika testing only:

- Urine
- Amniotic fluid

NOTE: Serum is the preferred diagnostic specimen. CSF, urine and amniotic fluid may only be tested alongside a patient-matched serum specimen.

SPECIMEN HANDLING AND STORAGE

- When transporting human specimens, ensure that all applicable regulations for transport of potentially infectious biological specimens are met.
- Transport/ship human specimens in dry ice, if possible; however, using cold-packs is acceptable.
- Store specimens at ≤-20°C upon receipt. Thaw sample and keep on ice during sample processing. Store remainder of sample at ≤-70°C for long term storage.

SAFETY/PRECAUTIONS

Laboratory biosafety guidance for working with Zika virus specimens is provided at http://www.cdc.gov/zika/state-labs/index.html. It is recommended that laboratories perform a risk assessment when conducting new tests and safety precautions should be based on the laboratory's risk assessment. Dengue and Zika viruses are considered pathogens that can be safely worked with in a biosafety level 2 (BSL-2) laboratory; however, according to the guidelines of the Biosafety in Microbiological and Biomedical Laboratories (BMBL), specimens suspected to contain chikungunya virus should be handled in BSL-3 conditions: http://www.cdc.gov/biosafety/publications/bmbl5/index.htm.

This procedure should be performed with consideration to the potential infectious nature of the specimens involved. The protocol is meant to detect viral genomes; therefore it is assumed that the specimens contain virus. Laboratorians should recognize that chikungunya virus produces high levels of viremia and specimens from suspected chikungunya virus cases should be treated as potentially infectious. Please review CDC guidance for state and local public health laboratories: http://www.cdc.gov/zika/state-labs/index.html. See the BMBL for additional biosafety information about these viruses and laboratory biosafety practices.

Proper disposable gown, gloves, eye protection, and a biological safety cabinet are recommended for manipulation of serum samples. The rRT-PCR assay should be performed in a separate room considered to be free of dengue virus (DENV), chikungunya virus (CHIKV), and Zika virus (ZIKV), or any virus RNA or DNA templates. Likewise, the RNA extraction procedure should be performed in a room different from where the RNA is amplified by rRT-PCR. During the nucleic acid amplification steps, sections of the viral genomes are amplified; therefore all original serum samples and test samples should be maintained separately from the PCR room to avoid contamination of samples.

Equipment and Consumables

DISCLAIMER: Names of vendors or manufacturers are provided as examples of suitable product sources. Use of trade names is for identification purposes only and does not constitute endorsement by CDC or the Department of Health and Human Services.

MATERIALS PROVIDED BY CDC

- CDC Trioplex Real-time RT-PCR Assay Primer and Probe Set (CDC; catalog #KT0166). Refer to product insert for storage and expiration information. Set includes 4 vials with primer and probes for each agent combined in one vial.
  - 1 vial, DENV-F, DENV-R1, DENV-R2, and P
  - 1 vial, CHIKV-F, R and P
  - 1 vial, ZIKV-F, R and P
  - 1 vial, RP-F, R and P (this is a primer/probe set for human RNAse P and is used to verify a successful extraction)
- Trioplex rRT-PCR Assay Positive Control Set (CDC; catalog #KT0167)
  - DENV Positive Control (PC): Inactivated dengue virus
  - CHIKV Positive Control (PC): Inactivated chikungunya virus
  - ZIKV Positive Control (PC): Inactivated Zika virus
  - Human Specimen Control (HSC): extraction control and positive control for RP

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA extraction kits (any of the following may be used):
  - MagNA Pure LC Total Nucleic Acid Isolation Kit (192 reactions) (Roche, catalog #03 03 85 05001)
  - MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, catalog #06543588001
  - Qiagen QIAamp® Viral RNA Mini kit (Qiagen catalog #52904 or 52906)
  - Qiagen QIAmp® DSP Viral RNA Mini kit (Qiagen catalog #61904)
- rRT-PCR Master mix kits (either may be used):
  - SuperScript® III Platinum® One-Step qRT-PCR Kit (ThermoFisher Scientific catalog # 11732088 and/or 11732020)
  - qScript™ One-Step qRT-PCR kit, Low Rox™ (Quanta, catalog # 95059-050 and/or 95059-200)
- Molecular-grade water, nuclease-free

EQUIPMENT

- Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument (ThermoFisher Scientific; catalog #446985 or #4406984);
- Vortex mixer
- Microcentrifuge
- 96 well cold block (or ice)
- Micropipettes (2 or 10 µL, 20 µL, 200 µL and 1000 µL)
- Multichannel micropipettes (5-50 µL)
- Automated RNA extraction instruments (optional):
  - MagNA Pure LC 2.0 instrument (Roche; catalog # 05197686001)
  - MagNA Pure 96 Instrument (Roche; catalog # 5195322001)

CONSUMABLES

- Acceptable surface decontaminants
  - DNA Away (Fisher Scientific; catalog # 21-236-28)
  - RNase Away (Fisher Scientific; catalog #21-236-21). This product eliminates RNase and DNA.
  - 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
  - DNA*Zap*™ (ThermoFisher Scientific; cat. #AM9890) or equivalent.
- Disposable, powder-free gloves and disposable gowns
- Laboratory marking pen
- Aerosol barrier sterile pipette tips for P2/P10, P40, P200, and P1000
- 1.5 mL microcentrifuge tubes
- Racks for 1.5 mL microcentrifuge tubes
- 0.1 mL PCR reaction plates (ThermoFisher Scientific; catalog #4346906 or #4366932) and optical caps (Applied Biosystems; catalog #4323032)
- MicroAmp® Optical Adhesive Film Kit (ThermoFisher Scientific; catalog # 4311971 or #4360954)

Quality Control

Real-Time RT-PCR is a sensitive method and should be conducted following strict quality control and quality assurance procedures. Following these guidelines will help minimize the chance of false-positive and false-negative results.

GENERAL CONSIDERATIONS

- Personnel must be familiar with the protocol and instruments used.
- Maintain separate areas, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips, gowns and gloves) for
  - assay reagent setup
  - handling of extracted nucleic acids

- Real-time RT-PCR amplification.
- Work flow must always proceed unidirectionally from the RNA extraction/reagent preparation (clean area) area to the PCR amplification room ("dirty area") in order to avoid contamination of clinical samples with amplified nucleic acids.
- Wear clean, previously unworn, disposable gowns and new, powder-free gloves during assay reagent setup and handling of extracted nucleic acids. Change gloves whenever you suspect they may be contaminated.
- Store primer/probes and enzyme master mix at appropriate temperatures (see package inserts). Do not use reagents beyond their expiration dates.
- Keep reagent tubes and reactions capped as much as possible.
- Use DNA*Zap*™ (or equivalent) or 10% freshly prepared bleach to clean surfaces.
- Do not bring extracted nucleic acid or PCR-amplified material into the assay setup area.
- Use aerosol barrier (filter) pipette tips only.

ASSAY CONTROLS

Assay controls should be run concurrently with all test samples.

Extraction control

Human specimen control (HSC) --- noninfectious cultured human cell material used as an extraction control and positive control for the RNase P primer and probe set (RP) that is **extracted concurrently** with the test samples and included as a sample during rRT-PCR set-up. The HSC should generate negative results with DENV, CHIKV and ZIKV primer and probe sets, but positive results for RP. The HSC is a component of the Trioplex rRT-PCR Positive Control Set (CDC; catalog #KT0167).

Positive controls for agent-specific primer and probe sets

- DENV PC: Inactivated dengue virus
- CHIKV PC: Inactivated chikungunya virus
- ZIKV PC: Inactivated Zika virus

These components of the Trioplex rRT-PCR Positive Control Set (CDC; catalog #KT0167) must be extracted using one of the acceptable RNA extraction methods described herein. Extracted positive nucleic acid should be aliquoted and stored at ≤-20°C until use. Avoid repeated freeze-thaw cycles.

Note: When extracting positive control material, precautions should be taken to prevent cross-contamination. Use caution when opening vials containing inactivated virus. Change or decontaminate gloves between each vial. Similar precautions should be observed when handling extracted positive control material.

RNase P Primer and Probe Set (RP)

All clinical samples and the HSC should be tested for human RNase P gene (using the RP primer and probe set included in the Trioplex rRT-PCR kit) to control for specimen quality and as an indicator that nucleic acid resulted from the extraction process. The RNase P Primer and Probe Set is a component of the Trioplex Real-Time RT-PCR Primer and Probe Set (CDC; catalog #KT0166).

No Template Control (NTC)

NTC reactions include PCR-grade water in place of specimen RNA and must be included for each reaction mixture (one for the ZIKV, CHIKV and DENV reaction and one for the RP reaction) in each run. The NTC is a control for contamination or improper function of assay reagents resulting in false positive results.

Table 1: Overview of positive and negative controls

Control Type	Control Name	Used to Monitor	DENV	CHIKV	ZIKV	RP	Expected C_T Values
Positive	DENV PC	Substantial reagent failure, including primer and probe integrity.	+	-	-	N/A	< 38 C_T
	CHIKV PC		-	+	-	N/A	
	ZIKV PC		-	-	+	N/A	
Negative	NTC	1) Reagent and/or environmental contamination during PCR set-up; and 2) primer and probe set function.	-	-	-	-	None Detected
Extraction	HSC	1) Reagent and/or environmental contamination during extraction; and 2) extraction success.	-	-	-	+	None Detected for DENV, CHIKV, and ZIKV.
							RP C_T < 38

Nucleic Acid Extraction

Notes on Extraction

- Sample extractions must yield RNA or total nucleic acid of sufficient volume to cover all real-time RT-PCR assays.
- Only acceptable specimens extracted using one of the prescribed extraction methods may be tested with this assay.
- HSC should be included in each extraction run as a sample extraction control.
- Retain specimen RNA extracts in cold block or on ice until testing.

Manual Extraction

Clinical specimens may be extracted using either the QIAamp Viral RNA Mini Kit or QIAamp DSP Viral RNA Mini Kit. Follow the manufacturer's instructions, using the following volumes:

Specimen input volume: 140µL
Elution volume: 60µL

Automated Extraction

- MagNA Pure LC 2.0 Instrument

  Clinical specimens may be extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit on the Roche MagNA Pure LC 2.0 extraction instrument using one of the Purification Protocols: Total NA_variable_elution_volume or Total NA External_lysis. Follow the manufacturer's instructions.

  Under the Total NA_variable_elution_volume protocol, use the following volumes:

  Specimen input volume: 200 µL
  Elution volume: 60 µL

  Under the Total NA External_lysis protocol, mix 200 µL of specimen with 300 µL of lysis buffer for a total volume of 500 µL before loading into the instrument.

  Elution volume: 60 µL

- MagNA Pure 96 Instrument

  Clinical specimens may be extracted using the MagNA Pure 96 Instrument using MagNA Pure 96 DNA or Viral NA Small Volume Kit. Make sure all reagents are equilibrated to room temperature before use. When setting up the instrument for an extraction run, use the DNA/Viral NA SV 2.0, then choose

"Viral NA Universal SV 3.0" or "Viral NA Plasma ext lys SV 3.0" purification protocol. Follow the manufacturer's instructions.

Under Viral NA Universal SV 3.0 protocol, use the following volumes:

Specimen input volume: 200 µL
Elution volume: 100 µL

Under the Viral NA Plasma ext lys SV 3.0 protocol, mix 200 µL of specimen with 250 µL of MP 96 lysis buffer for a total volume of 450 µL before loading into the instrument.

Storage of Nucleic Acid Specimens

Retain specimen RNA extracts in cold block or on ice until testing.

If testing will be delayed for more than 24hrs, freeze immediately at ≤ -20^{o}C. Only thaw the number of RNA extracts that will be tested in a single day. Do not freeze or thaw RNA extracts more than once before testing. For long term storage, > 7 days, freeze RNA extracts at -20^{o}C. RNA extracts stored at -20^{o}C should remain viable for 6 months.

Testing Algorithm

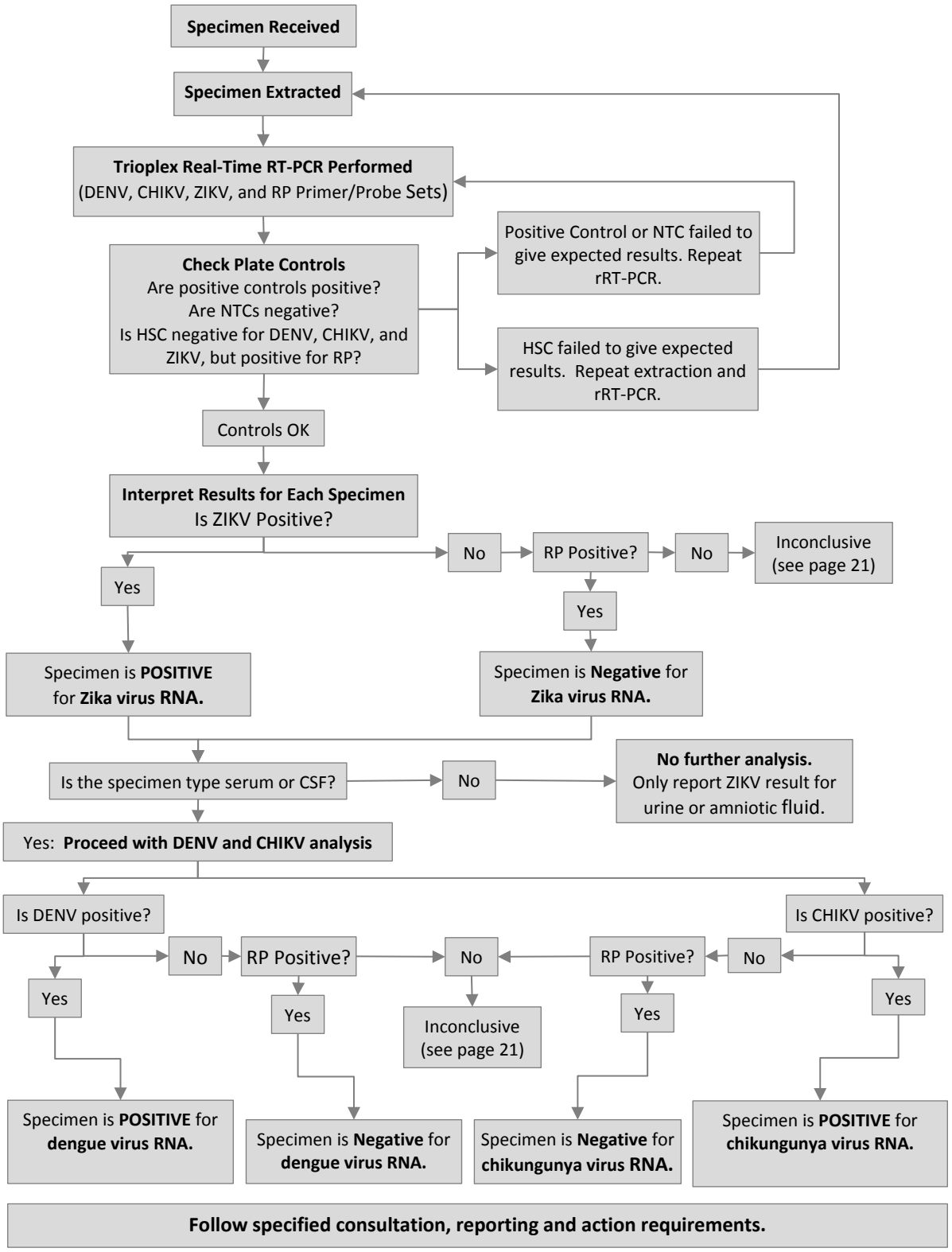


Figure 1: Summary of Test Results Interpretation

Real Time RT-PCR Assay

Stock Reagent Preparation

1. **Preparation of Real-time Primers/Probes**
    - Prior to rehydration, store kits at 2-8°C in the dark.
    - Precautions: These reagents should only be handled in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.
    - Carefully rehydrate lyophilized reagents in 250 µL of 10 mM Tris, pH 7.4 to 8.2 or PCR grade (nuclease free) water and allow to rehydrate for 15 min at room temperature in the dark.
    - Vortex each tube to obtain a uniform mix and aliquot primers/probe mix in 50 µL volumes into 5 pre-labeled tubes.
    - Store rehydrated aliquots of primers and probes at -20°C or below. Do not store in frost-free freezers.
    - Rehydrated primers and probes may be stored frozen for up to 24 months.
    - Thawed aliquots of probes and primers may be stored in the dark up to 4 months at 2-8°C during frequent use.
    - Do not re-freeze thawed aliquots.

Table 2: Primer and Probe Descriptions

Sequence Designator	Part Number	Gene Location
DENV-F	SO3684	5'-UTR
DENV-R1		
DENV-R2		
DENV-P		
CHIKV-F	SO3685	nSP1
CHIKV-R		
CHIKV-P		
ZIKV-F	SO3686	Envelope gene
ZIKV-R		
ZIKV-P		
RP-F	SO3687	Human Ribonuclease P
RP-R		
RP-P		

2. **Assay Controls**
   - Nucleic acid extracted from inactivated dengue virus
   - Nucleic acid extracted from inactivated chikungunya virus
   - Nucleic acid extracted from inactivated Zika virus

3. **No Template Control (NTCs)** (not provided)
   - Sterile, nuclease-free water
   - Aliquot in small volumes
   - Used to check for contamination during specimen extraction and/or plate set-up

4. **HSC extraction control**
   - Human Specimen Control must be extracted and processed with each batch of samples to be tested following the same procedure as with patient samples.
   - Do not dilute extracted RNA prior to testing

5. **Master mix**
   NOTE: Either **SuperScript® III Platinum® One-Step Quantitative RT-PCR System** or **qScript™ One-Step qRT-PCR kit, Low Rox ™** may be used.

   a. **SuperScript® III Platinum® One-Step qRT-PCR System**
      - Place 2X PCR Master Mix and Superscript III RT/Platinum Taq enzyme mix in a cold rack at 2-8°C.
      - Completely thaw the 2X PCR Master Mix vial.
      - Mix the 2X PCR Master Mix by inversion 10 times.

   b. **qScript™ One-Step qRT-PCR kit, Low Rox ™**
      - Thaw all components, except qScript One-Step RT, at room temperature.
      - Mix vigorously.
      - Centrifuge to collect contents to bottom of tube before using.
      - Place all components on ice after thawing.

Equipment Preparation

- Turn on Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument and allow the block to reach optimal temperature.
- Perform plate set up and select cycling protocol on the instrument (see Table 5).

Master Mix and Plate Set-up

Note: **Plate set-up configuration can vary with the number of specimens and work day organization. NTCs and assay controls must be included in each run.**

- In the reagent set-up room clean hood, place primer/probes on ice or cold-block. Keep cold during preparation and use.
- Thaw 2X Reaction Mix (SuperScript III or qScript) prior to use.
- Mix primer/probes by briefly vortexing.

- Briefly centrifuge primers/probes and return to ice or cold block.
- Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the NTC reactions and for pipetting error (see Table 2).
- Use the following guide to determine N:
  - If the number of samples (n) including controls equals 1 through 14, then N = n + 1
  - If the number of samples (n) including controls is greater than 15, then N = n + 2

Prepare reaction mixture according to the following table (**Table 3**). Keep reaction mixture on ice or in cold block.

Table 3: Trioplex rRT-PCR Reaction Mixture

TRIOPLEX Reaction Mix	
Component	**Quantity/Reaction (µL)**
Water	N x 0.5 µL
2x PCR Reaction Mix	N x 12.5 µL
DENV Mix	N x 0.5 µL
CHIKV Mix	N x 0.5 µL
ZIKV Mix	N x 0.5 µL
Enzyme Mix	N x 0.5 µL
Subtotal	**N x 15 µL**
Sample RNA	10 µL
TOTAL	**25 µL**

NOTE: The same reaction mixture volumes may be used for either the SuperScript III or qScript kit.

Table 4: RP PCR Reaction Mixture

RP Internal Control Reaction Mix	
Component	**Quantity/Reaction (µL)**
Water	1.5 µL
2x PCR Reaction Mix	12.5 µL
RP Mix	0.5 µL
Enzyme Mix	0.5 µL
Subtotal	**15µL**
Sample RNA	10 µL
TOTAL	25 µL

NOTE: The same reaction mixture volumes may be used for either the SuperScript or qScript kit.

PCR Plate Setup

a. In the reagent set-up room clean hood, while maintaining PCR plate on ice (or cold block), add 15 µL of reaction mixture to all wells being utilized.

b. Before moving the plate to the nucleic acid handling area, add 10 µL of nuclease-free water to the NTC wells
c. Loosely apply optical strip caps or optical tape to the tops of the reaction wells and move plate to the nucleic acid handling area on cold block or ice.
d. Remove optical strip caps or optical tape and add 10 µL of extracted sample RNA to each corresponding sample well. **Change tips after each sample addition.**
e. **Add 10 µL of DENV-1-4 Positive Control, CHIKV positive Control, ZIKV Positive Control and HSC (RP positive control) to separate wells as indicated in Table 5.**

Table 5: Example of Trioplex rRT-PCR plate layout for 3 samples

Mastermix Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Trioplex	Trioplex	Trioplex	Trioplex	Trioplex							
B	RP	RP	RP	RP	RP							
C												
D												
E												Trioplex
F												Trioplex
G												Trioplex
H												

Template Layout

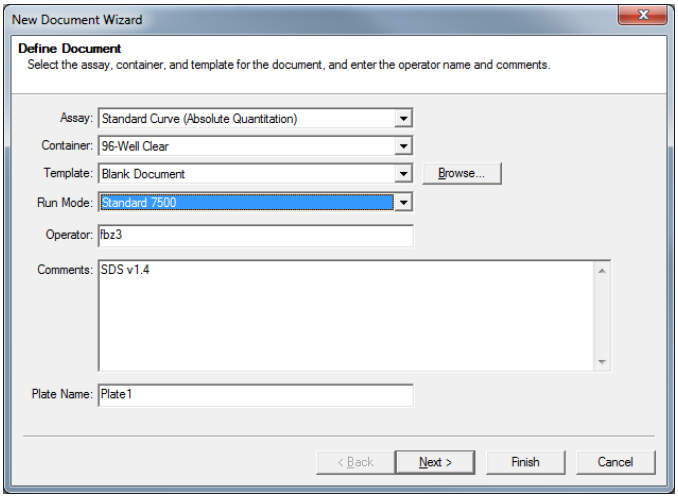
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	HSC	H2O NTC							
B	S1	S2	S3	HSC	H2O NTC							
C												
D												
E												DENV PC
F												CHIKV PC
G												ZIKV PC
H												

Positive controls: E12-H12

f. Seal plate with optical tape or caps and load plate on Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument.

2. **PCR Run**
   a. Launch the ABI 7500 software and select **Create new document**.
   b. Select **Standard 7500** on the Run Mode menu and click **Next** (**Figure 3**).

Figure 3: Select Run Mode



   c. Create a new detector for each target by clicking on **New Detector** (**Figure 4**)**,** name DENV, select reporter dye **FAM** and leave Quencher Dye as **none** **(Figure 5**).

Figure 4: Select Detector

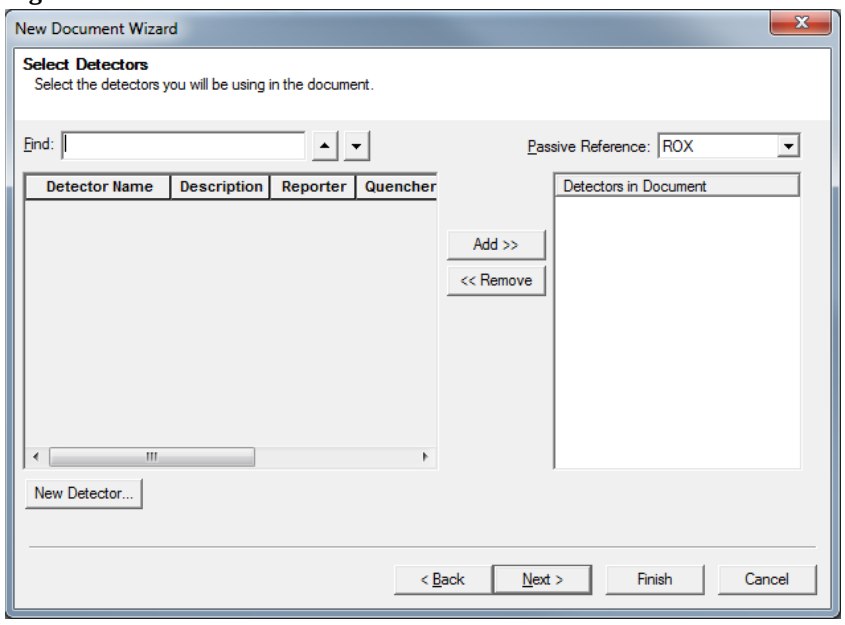
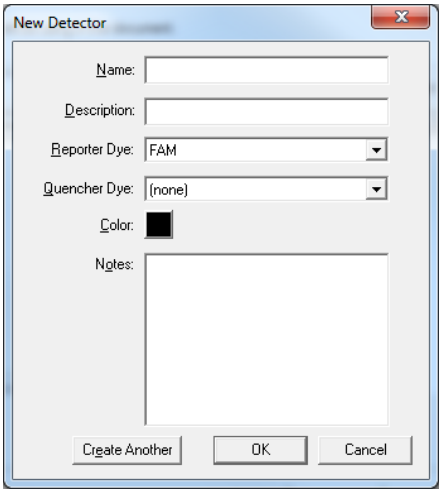
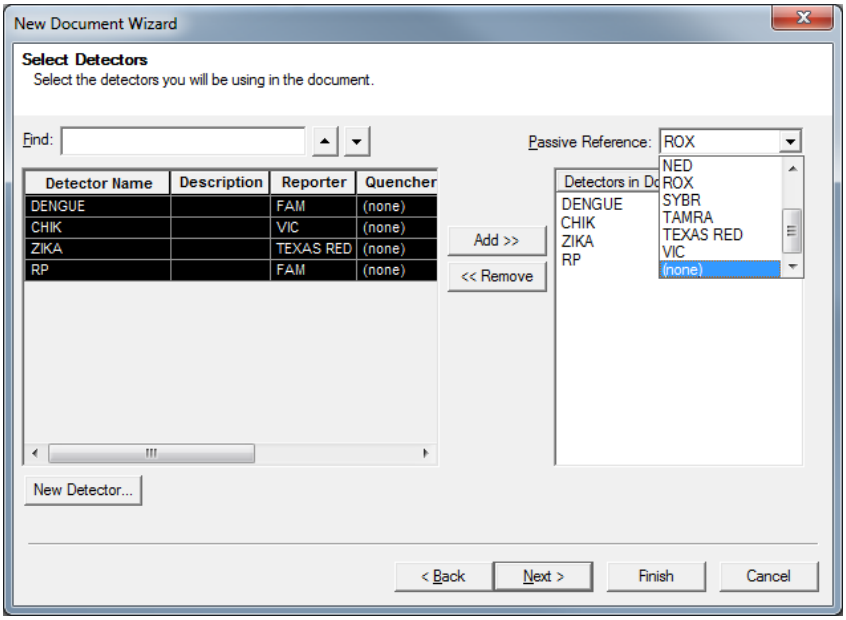


Figure 5: Reporter and Quencher Settings



d. Repeat for CHIKV, select reporter dye **VIC** and leave Quencher Dye **none**.
e. Repeat for ZIKV, select reporter dye **Texas Red** and leave Quencher Dye **none**.
f. Repeat for RP, select reporter dye **FAM** and leave Quencher Dye **none**.
g. On the Select Detectors screen, select **DENV** and click on **Add**.
h. Switch Passive Reference from **ROX** to **none (**Figure 6**).**

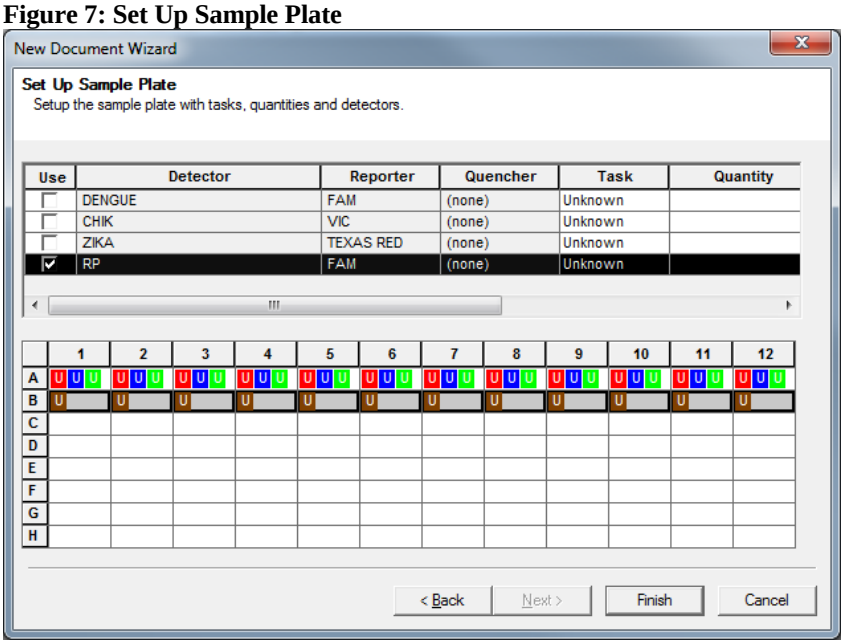
Figure 6: Passive Reference Selection



i. On the Select Detectors screen, select **CHIKV** and click on **Add**.
j. Switch Passive Reference from ROX to **none**.
k. On the Select Detectors screen, select **ZIKV** and click on **Add**
l. Switch Passive Reference from ROX to **none**.
m. On the Select Detectors screen, select **RP** and click on Add
n. Switch Passive Reference from ROX to **none**.

o. On the Set Up Sample Plate window, highlight corresponding wells and select **DENV**, **CHIKV**, and **ZIKV** detectors (**Figure 7**).

Figure 7: Set Up Sample Plate



p. Double click each well to enter sample name.

q. Select Instrument tab and define thermocycling conditions according to the master mix used:

(1) Stage 1: **30 min** at **50°C**; **1 rep**.

(2) Stage 2:

- SuperScript III: **2 min** at **95°C**; **1 rep**
- qScript: **5 min** at **95°C**; **1 rep**

(3) Stage 3, step 1: **15 sec** at **95°C**

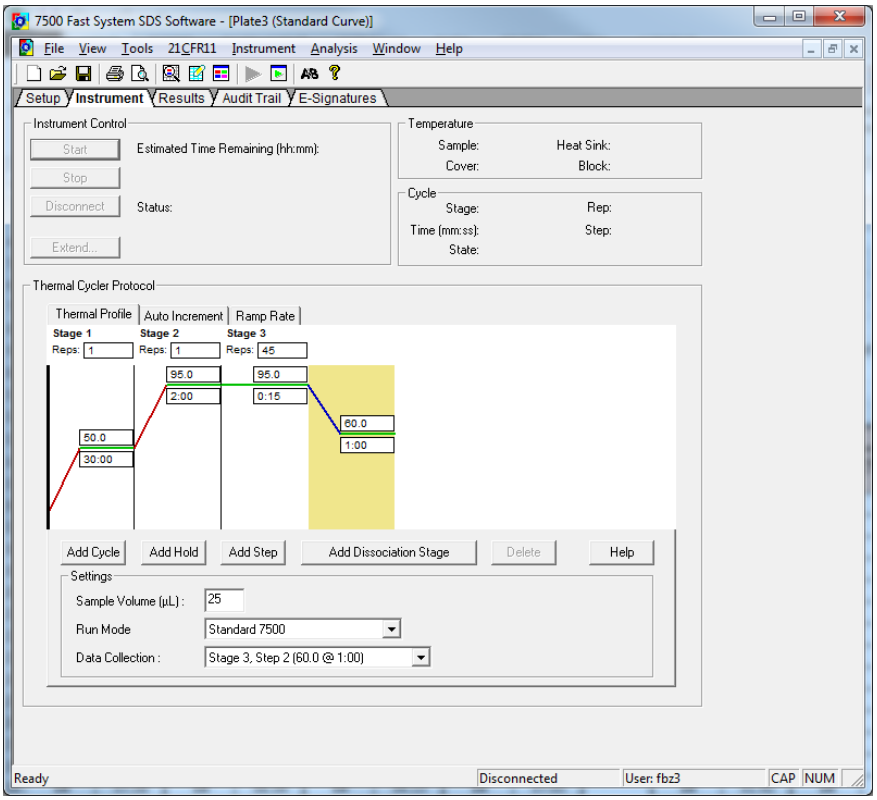
(4) Stage 3, step 2: **1 min** at **60°C**

(5) Stage 3: change reps to **45 cycles** (**Figure 8**)

SuperScript III Thermocycling Conditions			
STAGE 1	**STAGE 2**	**STAGE 3 STEP 1**	**STAGE 3 STEP 2**
30 min	**2 min**	15 sec	1 min
50°C	95°C	95°C	60°C
1 rep	1 rep		
		45 cycles	

qScript One-Step Thermocycling Conditions			
STAGE 1	**STAGE 2**	**STAGE 3 STEP 1**	**STAGE 3 STEP 2**
30 min	**5 min**	15 sec	1 min
50°C	95°C	95°C	60°C
1 rep	1 rep		
		45 cycles	

Figure 8: Set Thermal Cycling Conditions



(6) Under **Settings**, change volume to **25 µL**

(7) Under **Settings**, **Run Mode**, select **Standard 7500**

(8) Stage 3, step 2 should be highlighted in yellow indicating data collection

r. Select **Save As**, designate file name and folder

s. Click **Start**. Instrument will initialize and calculate time of run.

Data Analysis

After completion of the run, save and analyze the data following the instrument manufacturer's instructions. Analyses should be performed separately for each target using a manual threshold setting. Thresholds should be adjusted to fall within the beginning of the exponential phase of the fluorescence curves and above any background signal. The procedure chosen for setting the threshold should be used consistently.

Interpreting Test Results

Regardless of the specimen type being tested, prepare the PCR mix as indicated in "PCR Plate Setup."

TEST VALIDITY DETERMINATION

Before the results can be determined for each clinical specimen, the plate run must be determined to be **valid**. For a test to be valid, the controls must yield the expected results:

- Assay controls (nucleic acid extracted from inactivated DENV, CHIKV, and ZIKV) should be positive and within the expected C_T value range. If assay controls are negative
  - Repeat the plate.
  - If repeat testing generates a negative result from the positive control, contact the LRN helpdesk for consultation.
- NTCs should be negative. If NTCs are positive
  - Clean potential DNA contamination from bench surfaces and pipettes in the reagent setup and template addition work areas.
  - Extract and test multiple NTCs.
  - Discard working reagent dilutions and remake from fresh stocks.
  - Repeat samples only for the targets that are inappropriately amplified.
- HSC (extraction control) should be
  - Positive with RP primer/probe set due to the human DNA in the HSC
  - Negative with virus primer/probe sets. A positive result with the HSC and virus primer/probes would indicate cross-contamination has occurred. If a positive result is obtained, follow the cleaning procedure described above.
- RP Assay for each specimen should be **positive**.
  - If RP Assay for a specimen sample is *negative* and the Trioplex rRT-PCR assays are all *negative* for specimen samples:
    i. Report result as *Inconclusive* through LRN Results Messenger
    ii. Follow the instructions below:

All Specimen Types
1. Repeat rRT-PCR test of sample using RP and Trioplex assay.
2. Repeat extraction from new specimen aliquot if RP Assay is *negative* for specimens after repeat testing.
3. After repeat extraction and repeat rRT-PCR testing, if DENV, CHIKV, and/or ZIKV is *positive*, consider the result a true *positive* and continue to follow the testing algorithm.
4. If you are unable to resolve the results for a serum specimen, test other serum specimen tubes from the patient, if available, or request the collection of additional serum.

a. If RP Assay for a specimen sample is *negative*, but DENV, CHIKV, and/or ZIKV is *positive* for specimen samples:

- Do not repeat rRT-PCR test and consider the results of the Trioplex rRT-PCR valid.

If all controls have been performed appropriately, proceed to analyze each target.

NOTE: *The following section contains figures that are provided as generic examples. They are not specific to this assay.*

- True positives should produce exponential curves with logarithmic, linear, and plateau phases (**Figure 9**).
  (Note: Weak positives will produce high C_T values that are sometimes devoid of a plateau phase; however, the exponential plot will be seen.)

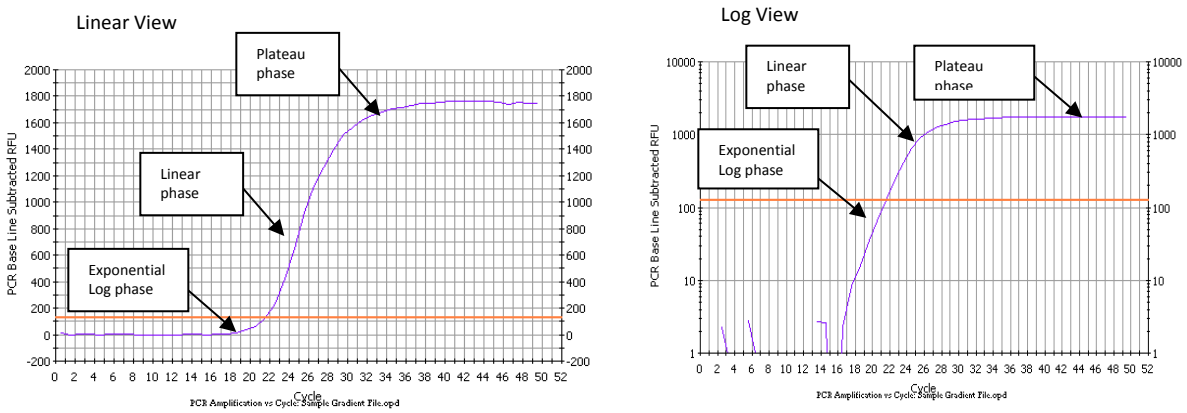


Figure 9: Linear and log views of PCR curves noting each stage of the amplification plots.

- For a sample to be a true positive, the curve must cross the threshold in a similar fashion as shown in **Figure 9**. It must NOT cross the threshold and then dive back below the threshold.
- **Figure 10** shows examples of false positives that do not amplify exponentially.

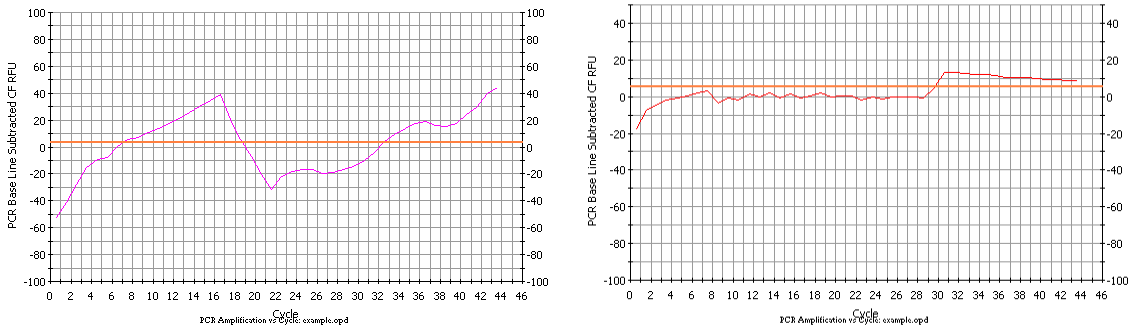


Figure 10: Examples of false positive curves.

- To better understand and evaluate challenging curves more effectively, use the background fluorescence view (Rn versus Cycle with AB software) to determine if the curve is actually positive. In this view, a sharp increase in fluorescence indicates a true positive while a flat line (or wandering line) indicates no amplification.
  - **Figure 11** shows a curve with a C_T value of 29.2 though it is evident that the sample is negative by looking at the background fluorescence view.
  - **Figure 12** shows an amplification plot with 3 curves: a moderately weak positive with a C_T of 36.6 (black), a very weak positive with a C_T of 42.1 (red), and a negative

control (blue). The weak positive (C_T= 42.1) is verified to be positive by the sharp increase in fluorescence seen in the background fluorescence view.

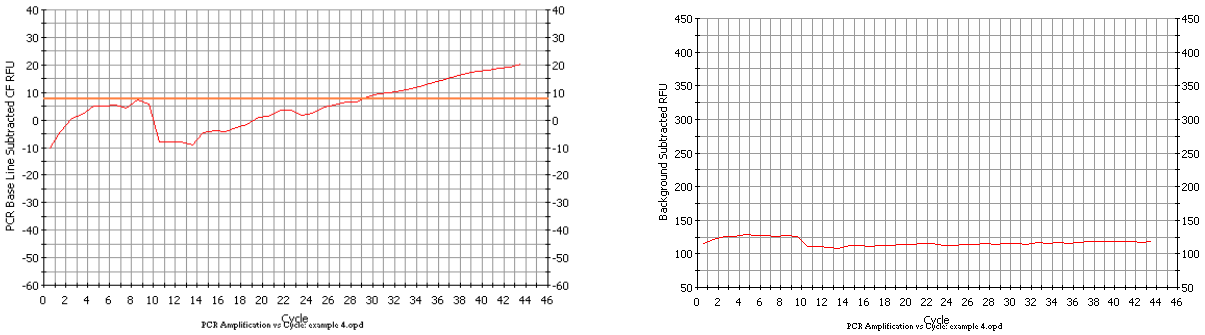


Figure 11: Amplification plot of a sample with a "wandering" curve (left) and the corresponding background fluorescence view (right).

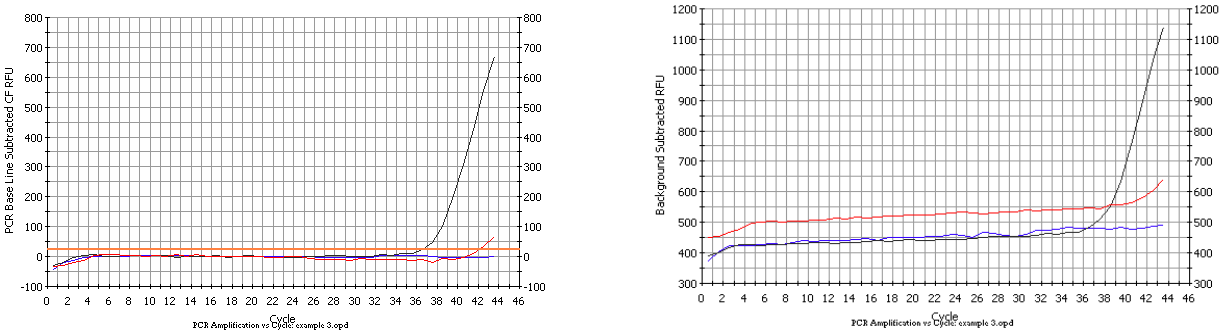


Figure 12: Amplification plot of three samples in the linear view (left) and the corresponding background fluorescence view (right).

- A note on weak positive samples: Weak positives should always be interpreted with caution. Look carefully at the fluorescence curves associated with these results. If curves are true exponential curves, the reaction should be interpreted as positive.
    - If repeat testing of a weak specimen is necessary, it is important to repeat the sample in replicates as a single repeat test run has a high likelihood of generating a discrepant result.
    - If re-extracting and re-testing the specimen, it may be helpful to elute in a lower volume to concentrate the sample.
    - The LRN helpdesk is available for guidance to help determine if repeat testing may be warranted and to discuss additional testing strategies as appropriate.

SPECIMEN INTERPRETATION AND REPORTING INSTRUCTIONS

All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.

The result generated for a primer and probe set is interpreted as positive if the reaction generates a fluorescence growth curve that crosses the threshold within (<) 38 cycles.

The result generated for a primer and probe set is interpreted a negative if:

- the reaction generates a fluorescence growth curve that crosses the threshold at or above (≥) 38 cycles, OR
- the reaction fails to generate a fluorescence growth curve that crosses the threshold.

Table 6: Trioplex rRT-PCR Interpretation and Reporting Instructions for Serum and CSF Specimens

ZIKV	DENV	CHIKV	RP	Interpretation	Reporting	Actions
-	-	-	+	Negative	No Zika, dengue, or chikungunya RNA detected by rRT-PCR	Report results to CDC. No further testing required. Note: If date of onset of symptoms is in doubt or if patient is asymptomatic, serological testing may be recommended. Refer to CDC algorithm.*
-	-	-	-	Inconclusive	Specimen inconclusive for the presence of Zika, dengue, and chikungunya RNA by rRT-PCR. An inconclusive result may occur in the case of an inadequate specimen.	Repeat extraction and rRT-PCR. If unable to resolve inconclusive result for a serum specimen, request collection of additional serum from the patient. Report inconclusive results to CDC.
-	+	-	+/-	Positive for DENV, but negative for ZIKV and CHIKV.	Dengue RNA detected by rRT-PCR. No Zika or chikungunya RNA detected.	Report results to CDC. Forward specimen to CDC. Refer to CDC algorithm.*
-	-	+	+/-	Positive for CHIKV, but negative for ZIKV and DENV.	Chikungunya RNA detected by rRT-PCR. No dengue or Zika RNA detected.	
+	-	-	+/-	Positive for ZIKV, but negative for DENV and CHIKV.	Zika RNA detected by rRT-PCR. No dengue or chikungunya RNA detected.	
-	+	+	+/-	Positive for DENV and CHIKV, but negative for ZIKV.	Dengue and chikungunya RNA detected by rRT-PCR. No Zika RNA detected.	
+	+	-	+/-	Positive for ZIKV and DENV, but negative for CHIKV	Zika and dengue RNA detected by rRT-PCR. No chikungunya RNA detected.	
+	-	+	+/-	Positive for ZIKV and CHIKV, but negative for DENV	Zika and chikungunya RNA detected by rRT-PCR. No dengue RNA detected.	
+	+	+	+/-	Positive for ZIKV, DENV, and CHIKV	Zika, dengue, and chikungunya RNA detected by rRT-PCR.	

* CDC Zika laboratory guidance and testing algorithm may be found on CDC's website: http://www.cdc.gov/zika/state-labs/index.html

If you have positive specimens to forward to CDC, please notify the LRN Helpdesk (LRN@cdc.gov) and request specimen shipment instructions.

Table 7: Trioplex rRT-PCR Interpretation and Reporting Instructions for Urine an Amniotic Fluid Specimens

ZIKV	RP	Interpretation	Reporting	Actions
-	+	Negative	No Zika RNA detected by rRT-PCR.	Report results to CDC. Refer to CDC algorithm.*
-	-	Inconclusive	Specimen inconclusive for the presence of Zika RNA by rRT-PCR. An inconclusive result may occur in the case of an inadequate specimen.	Repeat extraction and rRT-PCR. If repeat testing does not resolve inconclusive result, do not test further. Report results to CDC.
+	+/-	Positive	Zika RNA detected by rRT-PCR	Report results to CDC. Forward specimen to CDC. Refer to CDC algorithm.*

*CDC Zika laboratory guidance and testing algorithm may be found on CDC's website: http://www.cdc.gov/zika/state-labs/index.html

If you have positive specimens to forward to CDC, please notify the LRN Helpdesk (LRN@cdc.gov) and request specimen shipment instructions.

NOTE: All test results generated using the Trioplex rRT-PCR by LRN laboratories must be sent to CDC using LRN Results Messenger. Please refer to the LRN Data Messaging Policy (found under Documents/LRN Specific Information/LRN Policy Statements on the LRN website). For questions regarding this policy, please contact the LRN Helpdesk at LRN@cdc.gov.

Positive cases must also be reported through ArboNet.

NOTE: Please refer to the **Interpreting Test Results** section for detailed guidance on interpreting weak positives or questionable curves.

Assay Limitations

Interpretation of rRT-PCR test results must account for the possibility of false-negative and false-positive results. False-negative results can arise from:

- poor sample collection or
- degradation of the viral RNA during shipping or storage or
- specimen collection conducted prior to symptom onset
- specimen collection after nucleic acid can no longer be found in the patient (approximately 7 days post-onset of symptoms for sera)
- failure to follow the authorized assay procedures
- failure to use authorized extraction kit and platform

Application of appropriate assay controls that identify poor-quality specimens (such as RNase P) and adherence to CDC guidelines for DENV, CHIKV, and ZIKV testing can help avoid most false-negative results.

The most common cause of false-positive results is contamination with previously amplified DNA. Liberal use of negative control samples in each assay can help ensure that laboratory

contamination is detected and that false positive test results are not reported.

Negative results do not preclude infection with Zika virus and should not be used as the sole basis of a patient treatment/management decision. All results should be interpreted by a trained professional in conjunction with review of the patient's history and clinical signs and symptoms.

This assay is for *in vitro* diagnostic use under FDA Emergency Use Authorization only and is limited to qualified laboratories designated by CDC.

All specimens should be handled as if infectious. Proper biosafety precautions, including personal protective equipment, must be used when handling specimen materials. Additional information on safe handling of Zika virus specimens can be found at: http://www.cdc.gov/zika/state-labs/index.html.

Proper collection, storage and transport of specimens are essential for correct results.

Extraction of nucleic acid from clinical specimens must be performed with the specified extraction methods listed in this procedure. Other extraction methods have not been evaluated for use with this assay.

Performance has only been established with the specimen types listed in the Intended Use. Other specimen types have not been evaluated.

Performance Characteristics

Limit of Detection

The limits of detection (LoD) for the Trioplex rRT-PCR were established and re-verified over a number of studies. Findings for these are summarized in Table 8. LoD studies performed with MagNA Pure LC 2.0 Instrument (MP LC 2.0) and SuperScript III (SS III) are discussed in Sections 1.a. – 1.c. Other studies are discussed as bridging studies in Section 4.

Table 8: Overall limit of detection data summary

	Extraction	Mastermix	ZIKV (GCE/mL)	DENV (GCE/mL)	CHIKV (GCE/mL)
Serum	MP LC 2.0	SS III	1.54×10^4	2.15×10^4	3.99×10^5
	QIAamp	SS III	1.51×10^4		
	MP 96	SS III	1.95×10^4	1.48×10^4	3.79×10^5
	MP LC 2.0	qScript	3.11×10^4	3.86×10^4	8.05×10^5
Urine	MP LC 2.0	SSIII	1.79×10^4		
	QIAamp	SS III	2.15×10^4		
	MP 96	SS III	1.77×10^4		
	MP LC 2.0	qScript	4.20×10^4		

GCE = genome copy equivalent
MP 96 = MagNA Pure 96 Extraction Instrument

1. ZIKV LoD

   Limit of detection for the ZIKV primer and probe set was evaluated in both normal human serum and in urine using the French Polynesia 2013 strain of Zika virus. Five 10-fold serial dilutions in each matrix were prepared. For each matrix, each concentration was extracted 20 times using the MagNA Pure LC 2.0 Instrument and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results for serum are summarized in Table 9. Results for urine are in Table 10.

Table 9: ZIKV LoD in serum

Dilution	GCE/mL	ZIKV # Positive	Avg. C_T	DENV # Positive	CHIKV # Positive
1	1.93×10^7	20/20	28.10	0/20	0/20
2	1.25×10^6	20/20	31.58	0/20	0/20
3	9.62×10^4	20/20	34.84	0/20	0/20
4	1.54×10^4	20/20	37.17	0/20	0/20
5	1.54×10^3*	0/20	ND	0/20	0/20

* Based on concentration of dilution 4. In the absence of positive results at this concentration, quantification could not be determined for this dilution using the standard curve.
ND = not detected

Table 10: ZIKV LoD in urine

Dilution	GCE/mL	ZIKV # Positive	Avg. C_T	DENV # Positive	CHIKV # Positive
1	5.38×10^7	20/20	26.8	0/20	0/20
2	4.25×10^6	20/20	30.03	0/20	0/20
3	2.75×10^5	20/20	33.51	0/20	0/20
4	1.79×10^4	19/20	36.98	0/20	0/20
5	1.79×10^3*	0/20	ND	0/20	0/20

* Based on concentration of dilution 4. In the absence of positive results at this concentration, quantification could not be determined for this dilution using the standard curve.
ND = not detected

2. DENV LoD

Limit of detection for the DENV primer and probe set was evaluated in normal human serum using a representative strain from each dengue virus serotype (DENV-1 Puerto Rico 1998, DENV-2 Puerto Rico 1998, DENV-3 Puerto Rico 2004, DENV-4 Puerto Rico 1998). Five 10-fold serial dilutions of each strain in each matrix were prepared. For each matrix, each concentration was extracted 20 times using the MagNA Pure LC 2.0 Instrument and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results are summarized in Table 11.

Table 11: DENV LoD in serum

Dilution		GCE/mL	DENV # Positive	Avg. C_T	ZIKV # Positive	CHIKV # Positive
Serotype 1	1	5.82×10^5	20/20	32.55	0/20	0/20
	2	2.15×10^4	20/20	36.75	0/20	0/20
	3	3.44×10^3	1/20	39.08	0/20	0/20
	4	3.80×10^2	0/20	41.88	0/20	0/20
	5	38 *	0/20	ND	0/20	0/20
Serotype 2	1	8.25×10^6	20/20	29.18	0/20	0/20
	2	7.87×10^5	20/20	32.17	0/20	0/20
	3	1.72×10^4	19/20	37.03	0/20	0/20
	4	3.85×10^3	0/20	38.94	0/20	0/20
	5	3.85×10^2 *	0/20	ND	0/20	0/20
Serotype 3	1	4.36×10^6	20/20	29.99	0/20	0/20
	2	2.67×10^5	20/20	33.54	0/20	0/20
	3	1.66×10^4	20/20	37.07	0/20	0/20
	4	1.99×10^3	0/20	39.78	0/20	0/20
	5	1.99×10^2 *	0/20	ND	0/20	0/20
Serotype 4	1	2.68×10^6	20/20	30.61	0/20	0/20
	2	2.08×10^5	20/20	33.86	0/20	0/20
	3	1.36×10^4	19/20	37.33	0/20	0/20
	4	2.56×10^3	1/20	39.46	0/20	0/20
	5	2.56×10^2 *	0/20	ND	0/20	0/20

* Based on concentration of dilution 4. In the absence of positive results at this concentration, quantification could not be determined for this dilution using the standard curve.
ND = not detected

3. CHIKV LoD
   Limit of detection for the CHIKV primer and probe set was evaluated in normal human serum using the Puerto Rico 2014 strain of chikungunya virus. Five 10-fold serial dilutions in each matrix were prepared. For each matrix, each concentration was extracted 20 times using the MagNA Pure LC 2.0 Instrument and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results for serum are summarized in Table 12.

Table 12: CHIKV LoD in serum

Dilution	GCE/mL	CHIKV # Positive	Avg. C_T	ZIKV # Positive	DENV # Positive
1	1.28×10^7	20/20	31.95	0/20	0/20
2	1.30×10^6	20/20	35.42	0/20	0/20
3	3.99×10^5	19/20	37.22	0/20	0/20
4	2.01×10^5	2/20	38.26	0/20	0/20
5	2.01×10^4 *	0/20	ND	0/20	0/20

* Based on concentration of dilution 4. In the absence of positive results at this concentration, quantification could not be determined for this dilution using the standard curve.
ND = not detected

Inclusivity

1. DENV inclusivity evaluation
   Inclusivity of the DENV primer and probe set was evaluated using a panel of RNA from 29 international isolates of dengue virus, representing contemporary strains from all clinically relevant genotypes. Testing was conducted using the SuperScript III master mix. A summary of test results are in Table 13.

Table 13: DENV inclusivity across dengue viruses

Dengue virus serotype	Strains tested	DENV positive results
1	6	100%
2	11	100%
3	6	100%
4	6	100%

2. ZIKV, DENV and CHIKV sequence analysis
   In silico analysis of the Trioplex rRT-PCR primers and probes sequences was performed to verify reagent sequence homology with each corresponding virus and target region. A total of 514 current and historical dengue virus strains including 104 DENV-1, 142 DENV-2, 154 DENV-3 and 114 DENV-4, 206 chikungunya virus strains and 33 Zika virus strains were selected for this study. All primer and probe sequences showed 100% sequence identity with their expected target, predicting no false negative results are likely to occur. Table 14 below contains a summary of these findings.

Table 14: *In silico* inclusivity analysis

			Primer/Probe Sequence Identity									
Virus	**Strain**	**GenBank Acc#**	**DENV-F**	**DENV-R1**	**DENV-R2**	**DENV-P**	**CHIKV-F**	**CHIKV-R**	**CHIKV-P**	**ZIKV-F**	**ZIKV-R**	**ZIKV-P**
DENV-1	Mexico 2012	KJ189368	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-1	Nicaragua 2011	KF973453	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-1	Brazil 2010	JX669466	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-1	Saudi Arabia 2011	KJ649286	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-1	Thailand 2013	KF887994	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-2	Peru 2011	KC294210	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-2	Brazil 2010	JX669477	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-2	Indonesia 2010	KC762679	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-2	Saudi Arabia 2014	KJ830750	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-2	Singapore 2012	KM279577	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-3	Nicaragua 2009	JF937631	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-3	Indonesia 2010	KC762693	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-3	China 2013	KJ622195	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-3	Thailand 2010	HG316483	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-3	Brazil 2009	JF808120	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-4	Venezuela 2007	HQ332174	100%	<85%	100%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-4	Brazil 2010	JN983813	100%	<85%	100%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-4	Pakistan 2009	KF041260	100%	<85%	100%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-4	Singapore 2005	GQ398256	100%	<85%	100%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-4	Cambodia 2008	JN638570	100%	<85%	100%	100%	<20%	<20%	<20%	<20%	<20%	<20%
CHIKV	El Salvador 2014	KR559471	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Jamaica 2014	KR559489	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Trinidad & Tobago 2014	KR046231	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	French Polynesia 2015	KR559473	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Brazil 2014	KR264951	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Guyana 2014	KR559496	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%

			Primer/Probe Sequence Identity									
Virus	**Strain**	**GenBank Acc#**	**DENV-F**	**DENV-R1**	**DENV-R2**	**DENV-P**	**CHIKV-F**	**CHIKV-R**	**CHIKV-P**	**ZIKV-F**	**ZIKV-R**	**ZIKV-P**
CHIKV	Thailand 2013	KJ579186	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	India 2013	KT336782	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Indonesia 2013	KM673291	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	China 2012	KC488650	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Philippines 2013	AB860301	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Rep of Congo 2011	KP003813	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Singapore 2008	FJ445463	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Sri Lanka 2008	FJ513654	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	China 2008	GU199351	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Malaysia 2008	FJ807899	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	India 2008	JN558835	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Mexico 2014	KP851709	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Puerto Rico 2014	KR559474	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Honduras 2014	KR559488	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
ZIKV	China 2016	KU740184	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Brazil 2015	KU527068	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Guatemala 2015	KU501217	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Brazil 2015	KU365778	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	French Polynesia 2013	KJ776791	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Suriname 2015	KU312312	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Puerto Rico 2015	KU501215	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Thailand 2014	KU681081	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Philippines 2012	KU681082	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Martinique 2015	KU647676	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Micronesia 2007	EU545988	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Haiti 2014	KU509998	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	China 2016	KU744693	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
IKV	Brazil 2015	KU321639	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%

Exclusivity

1. Near-neighbor exclusivity evaluation
   Evaluation of the cross-reactivity of each component of the Trioplex rRT-PCR with the viruses targeted by the other components was evaluated extensively as a part of all LoD, bridging and contrived specimen evaluations. No cross-reactivity between the component primers and probes and these three viruses was observed.

   Three additional flaviviruses (WNV, YFV and SLEV) were selected to evaluate the specificity of the DENV, ZIKV and CHIKV primer and probe sets. Tissue culture supernatant of WNV (NY99 strain), YFV (17D strain), and SLEV (MSI-7 strain) were extracted with the Roche MagNA Pure LC 2.0 Instrument and tested using the SuperScript III master mix. All three viruses were tested in duplicate at 3 10-fold dilutions. No cross-reactivity was observed. All controls performed as expected.

Table 15: Near neighbor cross-reactivity

Virus	Strain	ZIKV result	DENV result	CHIKV result
West Nile virus	NY99	No cross-reactivity	No cross-reactivity	No cross-reactivity
yellow fever virus	17D	No cross-reactivity	No cross-reactivity	No cross-reactivity
St. Louis encephalitis virus	MSI-7	No cross-reactivity	No cross-reactivity	No cross-reactivity
Zika virus*	French Polynesia 2013	n/a	No cross-reactivity	No cross-reactivity
dengue virus*	Representatives from all 4 serotypes	No cross-reactivity	n/a	No cross-reactivity
chikungunya virus*	Puerto Rico	No cross-reactivity	No cross-reactivity	n/a

*Cross-reactivity findings for these three viruses were extrapolated from data presented in limit of detection, bridging, archived clinical specimen and contrived specimen evaluations.

2. Non-Arbovirus exclusivity evaluation
   A panel of viruses and organisms known to cause similar signs and symptoms to the viruses detected by the Trioplex rRT-PCR were selected for inclusion in an exclusivity evaluation. The nucleic acid was prepared from quantified stocks of qualified strains of each of the listed organisms. All organisms were tested in triplicate at one high concentration: 100pg nucleic acid/reaction. No cross-reactivity was observed. All controls performed as expected.

Table 16: Non-Arbovirus cross-reactivity

Organism		Concentration	Number positive		
			ZIKV	DENV	CHIKV
Bacteria	*Borrelia burgdorferi*	100pg/rxn	0/3	0/3	0/3
Fungus	*Histoplasma*	100pg/rxn	0/3	0/3	0/3
Protozoa	*Plasmodium falciparum*	100pg/rxn	0/3	0/3	0/3
Virus	Cytomegalovirus	100pg/rxn	0/3	0/3	0/3
	HSV-1	100pg/rxn	0/3	0/3	0/3
	Influenza A	100pg/rxn	0/3	0/3	0/3
	Influenza B	100pg/rxn	0/3	0/3	0/3
	VZV	100pg/rxn	0/3	0/3	0/3
	Vaccinia	100pg/rxn	0/3	0/3	0/3
	Adenovirus	100pg/rxn	0/3	0/3	0/3

3. *In silico* evaluation
   Additional evaluation of the analytical specificity of the Trioplex rRT-PCR was performed through *in silico* analysis of each primer and probe sequence against other common causes of acute febrile illness in humans. BLAST analysis queries of the Trioplex rRT-PCR primers and probes were performed against the GenBank public domain nucleotide sequences and showed no significant combined homologies (primer target and probe target) with other conditions that would predict potential false positive rRT-PCR results. Conditions and associated causative agents covered in the *in silico* specificity analysis are presented in Table 17.

Table 17: Organisms evaluated during *in silico* specificity analysis

Organism		Organism (taxid)
Bacteria	*Borrelia burgdorferi*	64895
	Group A Strep	36470
	Salmonella spp.	590
	Leptospira spp.	171
	Rickettsia spp.	780
Trematodes	*Schistosoma spp.*	6181
Fungus	*Histoplasma spp.*	5036
Protozoa	*Plasmodium falciparum*	5833

Organism		Organism
	Trypanosoma cruzi	5693
Flavivirus	Zika (DENV and CHIKV)	64320
	Dengue (ZIKV and CHIKV)	11052
	WNV	11082
	YFV	40005
	SLEV	11080
	Spondweni virus	64318
	JEEV	11071
Alphavirus	Chikungunya (ZIKV and DENV)	37124
	EEEV	11021
	WEEV	11039
	Ross River virus	11029
	Barmah Forest virus	11020
	O'nyong-nyong virus	11027
	Mayaro virus	59301
Other virus	Parvovirus (B19)	10789
	Measles virus	11234
	Rubella virus	11041
	Cytomegalovirus	10358
	HSV-1	10298
	HSV-2	10310
	Influenza A	11320
	Influenza B	11520
	VZV	10335
	Vaccinia	10245
	Epstein Barr virus	10376
	HIV	11676
	Hepatitis C	11102
	Enterovirus	12059
	Adenovirus	108098
		130310
		129951
		565302
		10519

Bridging Studies

1. qScript and Superscript III master mix evaluations:
   Four pools of material were prepared for evaluation: three pools of serum and one of urine. One pool of serum and one of urine were spiked with French Polynesia 2013 strain of Zika virus at the viral stock dilution factor identified as the LoD for ZIKV

with SuperScript III. One pool of serum was spiked with dengue virus (Puerto Rico 1998, serotype 2) at the viral stock dilution factor identified as the LoD for DENV with SuperScript III. And one pool of serum was spiked with chikungunya virus (Puerto Rico 2014) at the viral stock dilution factor identified as the LoD for CHIKV with SuperScript III.

Each pool was extracted using the MagNA Pure LC 2.0 Instrument 20 times. Each resulting RNA sample was tested by the Trioplex rRT-PCR using both the SuperScript III master mix and the qScript master mix. Results show comparable performance between the SuperScript III and qScript master mix. A standard curve was included in testing of each master mix. Both master mixes are acceptable for use with the assay. A summary of results is presented in Table 18 and Table 19.

Table 18: ZIKV qScript bridging summary

	ZIKV			
	Serum		**Urine**	
	SuperScript III	**qScript**	**SuperScript III**	**qScript**
AVG C_T	36.52	36.28	36.87	35.90
Std Dv	1.26	0.58	0.96	0.65
Positive	20/20	20/20	20/20	20/20
GCE/mL	**2.57×10^4**	**3.11×10^4**	**1.95×10^4**	**4.20×10^4**

Table 19: DENV and CHIKV qScript bridging summary

	DENV-2		**CHIKV**	
	Serum		**Serum**	
	SuperScript III	**qScript**	**SuperScript III**	**qScript**
AVG C_T	36.66	36.00	37.35	36.15
Std Dv	0.95	0.58	0.44	0.39
Positive	20/20	20/20	19/20	20/20
GCE/mL	**2.30×10^4**	**3.86×10^4**	**3.67×10^5**	**8.05×10^5**

2. MagNA Pure 96 Instrument extraction evaluation
   This study represented a repeat of the initial LoD study (Section M.1.) with the MagNA Pure 96 Instrument. The one difference is that a single dengue virus (Puerto Rico 2004, serotype 3) was selected for this evaluation instead of conducting the evaluation across all four serotypes. Results of the LoD evaluation for ZIKV are summarized in Table 20 and Table 21. DENV results are summarized in Table 22; CHIKV results are summarized in Table 23. The MagNA Pure 96 is acceptable for use with the assay.

Table 20: MagNA Pure 96 Instrument Evaluation – Zika in serum

Dilution	GCE/mL	ZIKV # Positive	Avg. C_T	DENV # Positive	CHIKV # Positive
1	2.71×10^6	20/20	30.60	0/20	0/20
2	2.94×10^5	20/20	33.42	0/20	0/20
3	1.95×10^4	19/20	36.87	0/20	0/20
4	6.36×10^3	2/20	38.30	0/20	0/20
5	6.36×10^2 *	0/20	ND	0/20	0/20

* Based on concentration of dilution 4. In the absence of positive results at this concentration, quantification could not be determined for this dilution using the standard curve.
ND = not detected

Table 21: MagNA Pure 96 Instrument Evaluation – Zika in urine

Dilution	GCE/mL	ZIKV # Positive	Avg. C_T	DENV # Positive	CHIKV # Positive
1	4.40×10^7	20/20	27.05	0/20	0/20
2	3.50×10^6	20/20	30.27	0/20	0/20
3	2.64×10^5	20/20	33.56	0/20	0/20
4	1.77×10^4	19/20	37	0/20	0/20
5	1.77×10^3 *	0/20	ND	0/20	0/20

* Based on concentration of dilution 4. In the absence of positive results at this concentration, quantification could not be determined for this dilution using the standard curve.
ND = not detected

Table 22: MagNA Pure 96 Instrument Evaluation – dengue in serum

Dilution	GCE/mL	DENV # Positive	Avg. C_T	ZIKV # Positive	CHIKV # Positive
1	1.43×10^5	20/20	34.34	0/20	0/20
2	1.48×10^4	19/20	37.23	0/20	0/20
3	1.53×10^3	0/20	40.11	0/20	0/20
4	1.53×10^2 *	0/20	ND	0/20	0/20
5	15.3 *	0/20	ND	0/20	0/20

* Based on concentration of dilution 3. In the absence of positive results at this concentration, quantification could not be determined for this dilution using the standard curve.
ND = not detected

Table 23: MagNA Pure 96 Instrument Evaluation – chikungunya in serum

Dilution	GCE/mL	CHIK # Positive	Avg. C_T	DENV # Positive	ZIKV # Positive
1	1.82×10^6	20/20	34.91	0/20	0/20
2	3.79×10^5	19/20	37.29	0/20	0/20
3	1.50×10^5	0/20	38.70	0/20	0/20
4	1.50×10^4 *	0/20	ND	0/20	0/20
5	1.50×10^3 *	0/20	ND	0/20	0/20

* Based on concentration of dilution 3. In the absence of positive results at this concentration, quantification could not be determined for this dilution using the standard curve.
ND = not detected

3. QIAamp extraction evaluation
   One pool of urine and one pool of serum were spiked using the French Polynesia 2013 strain of Zika virus to the dilution factor of viral stock identified as the LoD for ZIKV with the MagNA Pure LC 2.0 Instrument. Each pool was extracted 20 times using the Qiagen QIAamp DSP Viral RNA Mini Kit and tested with the Trioplex rRT-PCR using the SuperScript III master mix. Results support that the Qiagen QIAamp DSP Viral RNA Mini Kit performs in a non-inferior manner to the MagNA Pure LC 2.0 Instrument in the preparation of nucleic acid for subsequent testing by the Trioplex rRT-PCR and is acceptable for use in the assay.

Table 24: QIAamp extraction bridging summary

Matrix	Zika Virus GCE/mL	ZIKV # Positive	Avg. C_T	DENV # Positive	CHIKV # Positive
Serum	1.51×10^4	19/20	37.20	0/20	0/20
Urine	2.15×10^4	19/20	36.75	0/20	0/20

Clinical evaluation

1. Clinical performance of Trioplex rRT-PCR
   From the archival collection of the CDC Dengue Branch in Puerto Rico, 130 serum specimens were selected to evaluate the performance of the Trioplex rRT-PCR. Forty-eight (48) specimens from dengue cases (12 from each serotype), 12 from chikungunya cases, 20 from Zika cases and 50 negative specimens from symptomatic individuals were included in this specimen set. Upon removal from the archive (-70 ^oC), specimens were tested with the Trioplex rRT-PCR (using SuperScript III and the MagNA Pure LC 2.0 Instrument), the FDA-cleared DENV 1-4 rRT-PCR, singleplex in-house developed and validated Zika NS3 and chikungunya nSP1 rRT-PCR assays. Results of testing with the DENV 1-4 rRT-PCR, in-house Zika and chikungunya rRT-PCR assays matched the previous determination associated with all but one of the repository specimens.
   One archived Zika specimen generated negative results with the in-house Zika NS3 assay (C_T 38.45, assay positive cutoff <38) and positive Zika result with the Trioplex rRT-PCR. Due to the in-house Zika NS3 assay result, the archived Zika specimen was re-classified as a negative specimen and the Trioplex result analyzed as a false positive. Trioplex rRT-PCR results of testing are compared to this archival specimen category in the table below.

Table 25: Trioplex rRT-PCR performance with archived clinical specimens

Specimen category	Tested	Trioplex component result		
		ZIKV positive	DENV positive	CHIKV positive
Zika	19*	19/19	0/19	0/19
Dengue	48	0/48	47/48**	0/48
Chikungunya	12	0/12	0/12	12/12
Negative	51*	2/51	0/51	1/51
Positive percent agreement		**100%** (19/19) 95% CI: 83.2% - 100%	**97.9%** (47/48) 95% CI: 89.1% - 99.6%	**100%** (12/12) 95% CI: 75.8% - 100%
Negative percent agreement		**98.2%** (109/111) 95% CI: 93.7% - 99.5%	**100%** (82/82) 95% CI: 95.5% - 100%	**99.2%** (117/118) 95% CI: 95.4% - 99.9%

* One archived Zika specimen, when tested upon retrieval from archive, gave a C_T value just above the cutoff for the in-house Zika NS3 assay. Thus the specimen was re-classified as a negative specimen. The specimen gave a Zika positive result with the Trioplex assay, presented as a false positive result.
**One dengue specimen (serotype 4) generated a C_T of 39.87, which is a negative result. DENV 1-4 assay test result for the specimen was positive for serotype 4.

2. Secondary specimen data

Two urine specimens collected from symptomatic female patients suspected of Zika virus infection during the current Zika outbreak were submitted to the CDC laboratory in Puerto Rico for analysis. These specimens were tested with the Trioplex rRT-PCR using the MagNA Pure LC 2.0 Instrument and SuperScript III master mix. Specimens were also tested with the ZIKV primer and probe set run singleplex and with an in-house Zika NS3 rRT-PCR assay. Each specimen was tested alongside a patient-matched serum specimen collected the same day the urine was collected. Results are presented in Table 26.

Table 26: Urine specimen data

		Trioplex			ZIKV singleplex	Zika NS3 rRT-PCR
Case 1	DPO	DENV	CHKV	ZIKV		
Serum	3	Neg	Neg	32.51	33.21	34.6
Urine	3	Neg	Neg	29.34	28.56	31.56

		Trioplex			ZIKV singleplex	Zika NS3 rRT-PCR
Case 2	DPO	DENV	CHKV	ZIKV		
Serum	2	Neg	Neg	29.23	28.56	31.05
Urine	2	Neg	Neg	27.45	27.12	29.6

DPO = days post onset of symptoms

Amniotic Fluid and CSF Specimens:
Four amniotic specimens and two CSF specimens, all with Zika virus results from matched serum specimens, were evaluated with rRT-PCR analysis. The results from rRT-PCR analysis of amniotic and CSF specimens matched the serum Zika virus results in all cases.

3. Contrived specimen evaluation
Testing was conducted in two rounds. For the first round, 50 negative human serum specimens were used to prepare contrived specimens to evaluate the performance of the Trioplex rRT-PCR. Each specimen was aliquoted into 3 tubes. One aliquot from each specimen was not spiked (50 of specimen group 15). The remaining aliquots (n=100) were distributed into subgroups and spiked with whole virus as outlined in Specimen groups 1-13 in Table 29 below. For the second round of testing, an additional 25 contrived serum specimens were prepared: 15 as defined for specimen group 14, and 10 more negatives (specimen group 15) to mix in with them.

Low spiking level for Zika (French Polynesia 2013) was approximately 1.5-3 x LoD, moderate was approx. 100 x LoD, and high was approx. 1000 x LoD. For dengue (serotype 2, Puerto Rico 1998) and chikungunya (Puerto Rico 2014), low spiking level was 5-10 x LoD, high was 100-150 x LoD.

Aliquots were blinded and passed on to an operator for testing by the Trioplex rRT-PCR. Extraction was performed using the MagNA Pure LC 2.0 Instrument and rRT-PCR was conducted with the SuperScript II master mix. Results of testing are summarized in Table 28. Agreement between expected results and testing results for all three primer and probe sets was 100%.

Table 27: Spiking plan for contrived specimen study

Specimen group #	N	Zika	dengue	chikungunya
1	5	Moderate	-	-
2	5	-	Low	-
3	5	-	-	Low
4	5	High	-	-
5	5	-	High	-
6	5	-	-	High
7	10	Moderate	High	-
8	10	Moderate	-	High
9	10	High	Low	-
10	10	-	Low	High
11	10	High	-	Low
12	10	-	High	Low
13	10	High	High	High
14	15	Low	High	High
15	60	-	-	-

Table 28: Contrived specimen summary of results.

	High Positive		Moderate Positive		Low Positive		Negative	
	Tested	**Positive**	**Tested**	**Positive**	**Tested**	**Positive**	**Tested**	**Positive**
Zika	35	35	25	25	15	15	100	0
Dengue	50	50			25	25	100	0
Chikungunya	50	50			25	25	100	0

Procedure Notes

Send comments, suggestions and questions on this procedure to LRN@cdc.gov

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